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17. Supercritical fluid chromatography of lipids

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1 Introduction

Supercritical fluids (SF) have been used in separation science (extraction, chromatography and fractionation) for more than two decades. Supercritical carbon dioxide (SC-CO_2) has been the most popular fluid for use in extraction (SFE) and chromatography (SFC) in a wide spectrum of applications, including oils and fats in food and agricultural products, persistent organic pollutants in environmental samples, and pesticides in fruits and crops. Other fluids have been used as well, for example nitrous oxide, ethane, propane, pentane, ammonia, fluoroform, sulphur hexafluoride and water. However, in supercritical fluid chromatography (SFC) a clear majority of the published applications use SC-CO_2 with or without the addition of modifiers as mobile phase. The reason for the popularity of using CO_2 is its many advantages: it is non-toxic, inert, non-flammable, chemically stable, available in relatively high purity at a low cost, and it exhibits little interference using most detection modes.

Figure 1

A phase diagram of CO_2 is shown in Figure 1. A fluid is in its supercritical state at temperatures and pressures above its critical point. For CO_2, the critical temperature (T_c) is 31°C and the critical pressure (P_c) is 73 bar. Table 1 shows the critical parameters for other fluids as well.

Table 1

At supercritical fluid state, the distinction between the gas and the liquid phases has disappeared, and the resulting SF is of one uniform density (1). With increasing pressure, the density of a SF approaches that of a liquid solvent, giving it solvent properties equivalent to those exhibited by liquid media. In addition, SFs possess gas-like viscosities, which accelerate analyte mass transfer, and hence provide higher separation efficiency in SFC applications relative to LC. Therefore, SFC encompasses features of both LC and GC. Furthermore, the density of SFs and, hence their solvent strength properties, are easily changed by varying pressure and/or temperature. This enables the potential use of one fluid for chromatographic separation of many analytes of widely

differing polarity and molecular weight. This is in contrast to LC, where multiple liquid solvents are commonly used, confirming an advantage of SFC compared to LC.

However, the use of a single, SF, instead of several liquid solvents in a gradient-system is only one out of many advantages of using SFC. Use of SFC can result in faster separations than LC, due to higher mass transfer rate for solutes during the chromatographic process. Furthermore, when using SC-CO_2 as mobile phase, it is more environmentally compatible compared to LC, which consumes large volumes of organic solvents.

For example, Bicchi et al. (2) used PC-SFC with UV detection for analysis of valerenic acids and valepotriates in extracts from *Valeriana officinalis*. The qualitative and quantitative results of PC-SFC were comparable to HPLC for both valerenic acids and valepotriates, and analyses were faster when using PC-SFC than with HPLC. Figure 2 shows chromatograms of separation of valerenic acid, hydroxyvalerenic acid and acetoxyvalerenic acid using (A) PC-SFC and (B) HPLC, respectively, demonstrating the shorter retention times obtained with SFC relative to HPLC.

Figure 2

2 Principles of SFC

The most important parameters controlling retention in SFC are temperature, pressure, modifiers, and the flow rate of the SF. These parameters are described below. For additional information regarding the basic theory on SFC, the following references may be consulted (1,3,4).

2.1 Pressure and temperature

In SFC, both solute vapor pressure and solubility in the mobile phase contribute to the chromatographic retention. The solute's vapor pressure is the dominant factor at low SF density, while the solubility in the mobile phase is more significant at higher SF density (5).

As noted previously, pressure and temperature together determine the density of the SF. Increasing the pressure (at constant temperature) results in a higher mobile phase

density, and therefore a higher solvent strength of the SF. This relationship can be described by the modified Hildebrand equation (6) as:

Equation 1: $$\delta = 1.25\ P_c^{½}\ (\rho_{sf} / \rho_l)$$

Here the Hildebrand parameter, δ, is a measure of the solvent strength of a SF. P_c is the critical pressure of the SF, ρ_{sf} is the reduced density of the SF and ρ_l is the reduced density of a typical fluid in liquid state (~2.6-3.0).

The effect of temperature on the solvent strength of the SF is more complex, as it depends on whether the pressure is below or above the "crossover-point" for the solute's solubility in the SF (7). The solvent strength of the SF is decreased with increasing temperature, if the pressure is *below* the "crossover-point", simply due to the decreased density of the SF. However, the solvent strength of the SF is increased with increasing temperature, if the pressure is *above* the "crossover-point", despite the lower density of the fluid. This is due to the increasing vapor pressure of the analyte. This phenomenon is illustrated in Figure 3.

Figure 3

The "crossover point" has been determined for triacylglycerols (TAGs) (8) and plant-derived oils (9) for extractions using SC-CO_2. It is useful to know at which pressure the "crossover-point" occurs, as this controls the retention of the analytes. At the "crossover-point", the retention factor can reach a maximum, i.e. the longest retention time. A solute's solubility is inversely linearly proportional to the retention factor. Hence, retention in SFC has been used for providing a measure of the solubility of a solute in a SF under different conditions. Furthermore, retention data acquired on single capillary columns can be used for predicting retention data on coupled columns (10). In this same study (10), Karlsson et al. also described a second order relation between solute retention and mobile phase density. Clearly, both pressure and temperature programming can be useful tools in SFC for controlling solute retention.

2.2 Modifiers

Modifiers can be added to the SF to increase the solubility of the analytes. Some common modifiers that have been used with SC-CO_2 for SFC are methanol, ethanol, 2-propanol, dichloromethane and acetonitrile (2,11-13). In general, a modifier will strongly affect the retention in SFC. The small polar molecules interact with free silanol groups of the stationary phase, which thereby shortens the retention time of the analytes and improves their peak shape. A modifier may also change the order of elution of some solutes, as demonstrated for dimethyl phtalate in a mixture of biphenyls (14). In capillary SFC (described below under 3.1), organic modifiers cannot be used with flame ionization detector (FID), due to its large response to carbon-containing compounds.

2.3 Flow rate

The van Deemter curve describes the dependence of the height equivalent of theoretical plates (HETP) on the average flow rate (ν_0) of the mobile phase:

Equation 2 $$HETP = A + B/\nu_0 + C_m\nu_0 + C_{st}\nu_0$$

A is the Eddy diffusion term, B describes the longitudinal diffusion of the solute in the mobile phase, C_m describes the radial diffusion of the solute in the mobile phase, and C_{st} arises from the interactions of the solute with the stationary phase. Optimal (minimal) value of HETP can be obtained at a certain average flow rate of the SF. At this flow rate, the column has its highest separation efficiency under the selected conditions.

In SFC, the van Deemter curve is relatively flat after the minimum HETP and allows the use of high flow rates without significant losses in separation efficiency. This is of interest in preparative work, since faster flow rates leads to higher production rates.

3 Instrumentation

3.1 Major types

SFC instrumentation can be divided into two major categories: open-tubular SFC (OT-SFC, Figure 4A) and packed column SFC (PC-SFC, Figure 4B). OT-SFC has some similarities to capillary GC, while PC-SFC employs modified HPLC instrumentation. For

more information on instrumentation in SFC, the reader should confer the references (3,15-18).

Figure 4

In general, a SFC instrument consists of one or two high-pressure pumps (for delivery of SF and modifier if applicable), an injection device, a column placed in a temperature-controlled oven, a restrictor and a detector.

3.2 Supercritical fluid delivery

A high-pressure pump is necessary for delivering the SF to the column at very precise pressure. For capillary columns where the flow rate is low, a high-precision syringe pump capable of delivering a pulse-free flow of SC-CO_2 of pressures up to 500 bar is adequate. For packed columns where the flow rate is high, reciprocating pumps are more appropriate (5). Most pumps need to be cooled in order to achieve efficient filling with liquid CO_2.

3.3 Sample introduction

In general, sample injection in SFC is achieved using a high-pressure valve with an internal sample loop. The injection techniques can be divided into (i) direct injection; (ii) dynamic split injection; (iii) delayed split injection; and (iv) timed-split injection (3). The preferred injection technique depends on column type and sample amount/volume that needs to be injected.

In PC-SFC, regular HPLC columns with larger capacity for sample amount/volume are commonly used, and a direct injection technique may be used. In this injection mode, the entire volume of the sample loop is injected to the column, giving higher reproducibility compared to split-injection. Typically, volumes of up to1 μL are injected on columns of diameter 3 to 10 mm and length of 10 to 25 cm. In lipid applications, direct injection mode has widely been used (19-21).

The three latter injection techniques listed above [(ii) to (iv)] all concern different types of split-injection. These injection techniques are useful when narrow-bore and OT columns are used, since these columns have smaller capacities for large sample-volumes

(typically 1 to 10 nL). In dynamic and delayed split injection a restrictor device is placed downstream of the injector, very similar to common GC injectors. The injection volume is controlled by the ratio of the flow rates through the column and the split restrictor. The precision is better at high split ratios, but results in decreased sensitivity of the overall SFC method. In delayed split injection the split restrictor has an on/off valve, which initially is closed for a few seconds, and then opened to vent out the major part of the solvent. This technique is appropriate to use if larger volumes of sample are to be introduced into an OT column.

In timed-split injection, high-speed pneumatics and electronics control the injector, thus permitting a certain portion of the sample loop to be introduced onto the column. This injection mode offers higher precision and reproducibility as well as less sample discrimination compared to the other split-injection techniques. Timed-split injection is used in both OT and PC-SFC (13,22,23), and is the most commonly used injection technique in SFC for lipid analysis.

In any of the injection modes described above, it is important to carefully choose an injection solvent that is a good solvent for the sample and also compatible with the SF. However, there are options for solvent-less injection in SFC. One such way of introducing a sample onto a column is to couple a SFE system on-line to the SFC. On-line SFE/SFC has the advantage of less sample handling, less risk of sample contamination and no need for changing the sample solvent. Furthermore, it enables the injection of larger sample amounts since there is no liquid solvent present. This is especially useful in OT-SFC where only minute volumes of solvent can be tolerated. This type of extraction/analysis system has been used for analysis of fatty acids (FAs) and TAG mixtures (24), for oleic, linoleic and linolenic acid in whole-wheat flour (25), for cholesterol in egg yolk (24) and for analysis of fatty acid esters (FAEs) and polymer additives (26). The disadvantage with on-line SFE/SFC is that there are no commercial equipment available.

3.4 Columns

When choosing a SFC system, one of the first things to consider is which type of column is desirable for the intended application. OT columns offer outstanding efficiency

and inertness, while packed columns provide higher sample capacity and speed. OT columns inner diameter is typically 50 μm and their length 10 to 30 m, giving high efficiency (>3000 plates/min) and reasonable analysis time (<2 h) (5). In OT columns, the stationary phase must be cross-linked to the column surface to prevent the SF from dissolving the stationary phase material. For the separation of most lipids a non-polar stationary phase, such as a methyl polysiloxane is sufficient. That gives retention of solutes based on their molecular weight. For example, TAGs, free fatty acids (FFAs), squalene, α-tocopherol and cholesterol in fish oil were separated on a 5% phenyl-95% methyl polysiloxane column (20 m × 100 μm, 0.4 μm) (27). A more polar column, such as one having a cyanopropyl stationary phase, can be useful for separation of FAs and acylglycerols of equal carbon number differing in the number and position of double bonds. This was demonstrated by Manninen et al. who separated TAGs from alpine currant and black currant seed oils on a 25% cyanopropyl-75% methyl polysiloxane column (28) and TAGs from berry oils on a 25% cyanopropyl-25% phenyl-50% methyl polysiloxane column (29).

In OT-SFC, the primary variables affecting retention are the density of the mobile phase and the polarity of the stationary phase (30). In PC-SFC on the other hand, retention is influenced mainly by the properties of the stationary phase and polarity of the mobile phase. IN PC-SFC, the stationary phase is usually based on silica particles, either unreacted or chemically modified (30). Even if modified with C18 carbon chains, there are always some unbonded silanol groups present. Polar molecules will interact with these silanol groups, resulting in long retention times and deformed peak profile. However, the addition of a modifier to the unpolar SF leads to shorter retention times and improved peak shapes. For example, France et al. showed that the addition of water to SC-CO_2 in PC-SFC improved the peak shapes of FAs (21). The use of water as a modifier also has the advantage of allowing the use of FID.

Lesellier and Tchapia showed that the retention of TAGs on octadecyl packed columns in SFC was similar to non-aqueous reversed phase HPLC (12). Figure 5 demonstrates that the retention order of TAGs is dependent on the carbon number (CN) and the unsaturation number (UN) of the solute; higher CN and lower UN resulting in

longer retention times. The same type of observation was made for TAGs in turnip and rapeseed oil using a Nucleosil C18 5-μm column (100 mm × 1 mm i.d.) and SC-CO_2 as mobile phase (19). (See Figure 5).

Figure 5

Retention of FAMEs on aminopropyl-bonded silica columns with different stationary phase bonding densities was studied by Sakaki (31). It was shown that retention depended on both CN and UN. At higher aminopropyl bonding density retention of FAMEs mostly depended on their CN (molecular weight), while at lower aminopropyl bonding density, retention of FAMEs were more determined by their degree of unsaturation (UN).

Smith et al. (20) demonstrated that retention of FAMEs on packed unbonded silica columns in SFC is determined by the UN only, and not by the CN. In fact, they found a positive linear relation between retention time and number of cis double bonds for FAMEs on Spherisorb S5 W silica columns using SC-CO_2 as mobile phase.

3.5 Flow restrictors

The flow restrictor is important for controlling the pressure over the column, mainly to maintaining a constant density along the length of the entire column as well as constant flow rate during SFC analysis. The flow restrictor is attached to the end of the column, either before or after the detector. If the restrictor is placed after the detector, a high-pressure cell is required. This is common with UV/VIS, diode array detection (DAD) and fluorescence detectors. In FID, nitrogen-phosphorus detection (NPD), flame photometric detection (FPD) and MS, the restrictor is placed before the detector. The ideal restrictor should not get plugged, or discriminate towards certain analytes, and the flow rate should easily be adjusted by changing the length or diameter of the restrictor. For FID, NPD, FPD and MS, where both flow restriction and rapid decompression to atmospheric pressure occur before the detector, the design of the restrictor is rather crucial (5). These capillary restrictors can be designed as straight, tapered and frit-type options, the latter showing the most promising properties in terms of avoiding sample discrimination and condensation of analytes on the inside of the restrictor.

3.6 Detection

Most detectors used with GC (FID (21,32), NPD (33), FPD (34), electron capture detection (35) and ion-mobility detection (36)), with HPLC (UV (37,38), diode array (39), ELSD (40), mass evaporative detection (20), electrochemical detection (41) and fluorescence detection (42)), as well as MS (43) and Fourier transform IR detection (44), have been interfaced with SFC. In general, PC-SFC is frequently coupled to UV and fluorescence detectors. FID can also be used, but because of the high flow rates produced in PC-SFC (around 20 ml/min), splitting of the column flow is necessary, which results in lower analyte sensitivity. Furthermore, FID cannot be used in combination with most modifiers as noted previously. In OT-SFC, FID is the most common detector for lipid analysis, but MS is becoming more and more common. The advantages of using MS detection in SFC are its mobile phase independence, high sensitivity and capability of providing structural information.

Markides and her previous colleagues at Brigham Young University in Utah and her present research group in Uppsala University, Sweden, have made considerable effort developing good interfaces for capillary SFC/MS (43,45-49). A capillary SFC was coupled to a high resolution double focusing MS by using a frit restrictor for reducing the pressure and a direct insertion probe for heating the restrictor (46). A similar study was reported using the same OT-SFC/MS system, gave excellent mass spectra of high-molecular weight compounds at the low nanogram levels (45). Bücherl et al. (26) also coupled SFE-SFC/FID to a high resolution double focusing MS, and used the system for analysis of FAEs and polymer additives.

In a more recent study by Sjöberg and Markides (47), a SFC interface probe for atmospheric pressure ionization MS (API-MS) was constructed, which allowed easy switching between electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) modes. In a similar study (43), this interface was further improved by mounting the corona needle directly on the interface probe, and by heating the restrictor tip to compensate for the effect of adiabatic cooling of the expanding CO_2, thereby improving the detection limits by a factor 20-25 in the 50-0.1 pg range.

4 Applications

4.1 Lipid classes

A main characteristic of SFC is its ability to separate compounds of widely varying polarity and molecular weight within the same chromatographic run. Usually, it would take several independent methods of analysis to determine components such as FFAs, steroids, wax esters and TAGs. There are several examples in the literature showing the separation of lipids in complex samples using a single run of SFC. Some of these applications have been summarized in Table 2.

Table 2

King (50) used OT-SFC for separation of cholesterol from TAGs in fish oil, lipophilic compounds in a lipstick formulation, long chain FAs and alcohols in saponified jojoba oil and wool wax esters and polyesters in a hydrogenated lanolin sample. These applications certainly demonstrate the versatility of the SFC technique. Figure 6 shows a chromatogram for analysis of lipid compounds in lipstick.

Figure 6 from Jerry (lipstick)

Staby et al. (22,51) used a nonpolar OT-SFC with FID for group separation of FFAs, retinol, ergocalciferol, cholecalciferol, squalene, tocopherols, cholesterols, wax esters, diacylglycerols (DAGs), cholesteryl esters and TAGs in different marine oils. The total analysis time was 120 min, which is acceptable keeping in mind that the alternative is to perform both GC and HPLC with foregoing sample derivatization. The chromatographic separation of this complex mixture of lipid components is shown in Figure 7. In a similar study from the same team in Denmark (52), shark liver oils were analyzed by OT-SFC with FID. It was shown that squalene and cholesterol could be quantified by the SFC method, while TAGs, cholesterol esters and DAG ethers required thin-layer chromatographic fractionation prior to SFC analysis.

Figure 7

SFC can be a useful tool in the monitoring of reactions involving lipids. For example, Hayes et al. (53) used a non-polar SB-methyl-100 (10 m $\times$ 50 μm ID) column

with FID for studying *Dimorphotheca pluvialis* oil that was exposed to lipase-catalyzed hydrolysis and alcoholysis. FAs, FAEs, mono- di- and triacylglycerols and estolides were easily separated by groups.

The benefits of using SFC for monitoring purpose are even more obvious when studying reactions conducted in SC-CO_2. Temelli and colleagues used SFC on a routine basis for studying lipase-catalyzed conversion of canola and soybean oils to high-value products such as mono- and diacylglycerols (23,54-56). The reactions were performed in SC-CO_2 in a stirred autoclave. Analyses were accomplished on OT-SFC (SB-Octyl-50 capillary column, 10 m × 100 μm ID, 0.5 μm film thickness) and FID. SC-CO_2 was used as the mobile phase with a pressure program starting at 120 atm for five minutes, then increase to 300 atm at a rate of 8 atm per min and final hold of three minutes (54). The total analysis time was 30 minutes. A representative chromatogram is shown in Figure 8.

Figure 8

4.2 Triacylglycerols

A majority of the SFC applications on lipids concern the separation of TAG species. A large selection of these applications is summarized in Table 3.

Table 3

Separation of TAGs by OT-SFC has been thoroughly studied by Manninen, Laakso and Kallio at the University of Turku in Finland (28,29,57-60). They showed that γ- and α-linolenic acid-containing TAGs of identical CN and UN could be separated on a 10 m × 50 μm 25% cyanopropyl-75% methyl polysiloxane column using SC-CO_2 as mobile phase and FID for detection (28). A similar study was performed on a 25% cyanopropyl-25% phenyl-50% methyl polysiloxane column for the separation of TAGs in various berry oils and complex milk fat and fish oil samples (29). Retention of TAGs in these polar columns depends on both the components different CN and UN as discussed above. A representative chromatogram is shown in Figure 9.

Figure 9

In some more recent studies, Manninen and Laakso (57,58) coupled a OT-SFC system with a triple-quadrupole mass spectrometer (MS) via a LC-APCI interface, and used this system for analysis of TAGs in various berry oils. In both investigations a 25% cyanopropyl-25% phenyl-50% methyl polysiloxane column (10 m × 50 μm i.d.) was used. The SFC/MS system was more appropriate for identifying and quantifying TAGs than those SFC/FID systems described above, as the MS is more sensitive than FID, and in addition gives some structural information. In another study from the same research group, using OT-SFC/MS system analyzed milk fat TAGs (60). It was found that only saturated TAGs give rise to $[M+18]^+$ ions, which was very useful for structure elucidations.

Hannan and Hill, Jr (32) determined the composition of TAGs in aging onion seed using OT-SFC/FID on a non-polar column. They showed that the retention time and quantitative response of the lipids was very reproducible. Demirbüker et al. (61) used micropacked argentation PC-SFC for the analysis of TAGs in the seeds of *Aquilegia vulgaris*. Columns were prepared from fused silica (330 mm × 250 μm i.d.) and packed with Nucleosil 5 SA and rinsed with silver nitrate. The mobile phase consisted of SC-CO_2/acetonitrile/2-propanol (92.8:6.5:0.7, mol%) and UV detection was used. Analysis time was less than 60 min.

Borch-Jensen et al. (27) determined the composition of lipids in fish oil using both non-polar and polar OT-SFC/FID. It was demonstrated that the non-polar column separated the TAGs based on molecular weight only, while the polar column enabled the separation of several more of the TAGs based on both molecular weight and degree of unsaturation. Moreover, the non-polar column was useful for analysis of in addition to TAGs also FFAs, squalene, α-tocopherol and cholesterol.

Shen et al. (62) used a home-built SFC for analysis of TAGs in tea oil, cod liver oil, rancid butter and some Chinese medicines. Good separation was achieved on the fused silica capillary column (16 m × 100 μm) coated with a 0.20 μm film of cross-linked polymethylsiloxane.

Several studies describe the use of SFC for the analysis of structured lipids (63,64). Klemann et al. (63) employed a 100 mm × 1 mm Deltabond Cyano 5-μm column and

FID for analysis of interesterified short-chain and long-chain fatty acid TAGs. The composition of the oil was found to be in good agreement with values calculated by a statistical model for random interesterification. Lee and Hastilow (64) also used SFC for characterization of structured lipids. They used a SB-methyl-100 capillary column (10 m × 100 μm i.d.) with FID for separation of TAGs with respect to their equivalent CN. TAGs with three carbon number differences were very well resolved by both the SFC method and a high-temperature GC method. However, the SFC method did not require any sample pre-treatment, while the GC method necessitated hydrogenation of the oil sample. The SFC method was also applied to the analysis of a fish oil sample.

Artz et al. (65) used polar OT-SFC for determination of the decreasing amount of TAGs in heated triolein and trilinolein oil. They also studied the degradation of oleic acid-esterified propoxylated glycerol (EPG-08 oleate) and EPG-08 linoleate using nonpolar OT-SFC. The results showed that less than 40% of the original trilinolein remained at the end of the 24-h heating treatment at 190°C, compared to 78% as measured by high-performance size-exclusion chromatography (HPSEC). It was concluded that SFC is a more accurate technique than HPSEC for analyzing degradation of oil during heating.

Hayes and Kleiman (66) determined the oil constituents in *Lesquerella fendleri* seeds containing TAGs rich in hydroxyfatty acid. The oil was fractionated on a silica gel column and then analyzed by non-polar OT-SFC/FID. In another study by the same authors (67), TAGs from crambe, meadowfoam, Euphorbia lagascae and vernonia oils were analyzed on the same non-polar OT-SFC/FID system. Both pressure and temperature programming was applied, and the method was used for separation of reaction products in lipase-catalyzed transesterification reactions involving estolides and the oils listed above. Analyte molecular weights were estimated from a retention time/molecular weight calibration curve.

4.3 Free fatty acids

A few applications describe the use of SFC for separation of molecular species of FFAs. Applications on the use of SFC for separation of FFAs and FA derivatives have been summarized in Table 4.

Table 4

In general, the strength in SFC lies in the fact that FFAs can be separated in the same run as mono- di- and triacylglycerols, and other lipids as well (see above under Lipid Classes). An interesting application of SFC is for detection of by-products in lipid samples formed during processing or storing. Skovly et al. (68) analyzed a technical product of saturated FAs for trace amounts of by-products formed by C20-22 FAs. They used OT-SFC (10 m × 50 μm i.d. Dionex SB-Biphenyl-30 column,) with FID and MS detection for determination of the FA components. It turned out that the by-products were a series of homologs of FA dimers, identified as lactone esters. A chromatogram is shown in Figure 10.

Figure 10

Borch-Jensen and Mollerup used polar OT-SFC/FID for determination of vernolic acid content in *Euphorbia lagascae* oil and compared the SFC technique with GC (69). Two different sample pre-treatments were applied for SFC analysis: (i) preparation of FFAs by saponification of the oil, followed by injection into the SFC; and (ii) straight injection of the raw oil into the SFC. The FA results were very similar, but compared to base-catalyzed transmethylation of the oil followed by GC analysis, the SFC results were slightly higher. This was explained by the fact that base-catalyzed treatment will not methylate the 2% of free vernolic acid occurring in the oil. Hence, the SFC method was more accurate than the GC method.

In another similar study by Borch-Jensen et al. (70), the seed oil from *Ricinus communis* and *Dimorphoteca pluvialis* were analyzed for their hydroxy FA content. The oil was either injected directly into the polar OT-SFC/FID or first hydrolyzed to FFAs before injection to the same SFC system. The obtained results compared well with a GC method that included formation of TMS-FAMEs of the hydroxy FAs prior to GC

analysis. This study clearly demonstrates one of the advantages of SFC: by simply changing the density program, the same fluid (SC-CO_2) and column can be used for separating TAGs in one run and FFAs the next run.

Bicchi et al. (2) used PC-SFC/UV for analysis of valerenic acid and valepotriates in *Valeriana officinalis* root extracts. Several packed columns were tested, Lichrospher 100 RP-18 (C18; 250 × 4 mm i.d., 5μm), Lichrosorb RP-8 (C8; 250 × 4 mm i.d., 10μm), Lichrosorb Diol (Diol; 250 × 4 mm i.d., 5μm), Silica Spherisorb (Sil; 150 × 4.6 mm i.d., 3μm), and S3-Nitrile Spherisorb (CN; 150 × 4.6 mm, 3μm). Various modifiers were also tested, including methanol, methanol/water, ethanol, isopropanol, chloroform, and acetonitrile. SC-CO_2 containing 0 to 2.5 vol% of methanol/water (95:5) was found to be the best mobile phase, and a column packed with CN-modified stationary phase, gave the best separation of valerenic acid and valepotriates. The SFC method was compared to a common HPLC method for this application, and it was demonstrated that the optimized SFC method gave much faster analysis time than the corresponding HPLC method.

4.4 Fatty acid derivatives

FAMEs and fatty acid ethyl esters (FAEEs) have been analyzed using SFC, and some of the applications are shown in Table 4.

Table 4

PC-SFC/UV was used for the analysis of FAMEs in fish oil (13). Three different types of column packing were tested: cation exchanger impregnated with silver nitrate, untreated silica, and an anion exchanger treated with potassium permanganate. SC-CO_2 containing acetonitrile and isopropanol was used as mobile phase. The results showed that the silver nitrate column gave better resolution of the fish oil FAMEs than the silica packed column. The permanganate-treated anion exchanger separated the FAMEs into groups according to their UN.

Sakaki (31) also used PC-SFC/UV for separation of FAMEs in fish oil. Different stationary phase bonding densities of aminopropyl-bonded silica were tested using standard mixtures of FAMEs. The results showed that separation according to CN was dominant at high aminopropyl bonding density, while separation according to UN was

dominant at low aminopropyl bonding density. FAMEs in fish oil were better separated on aminopropyl-bonded silica packings of high bonding density.

Staby et al. (71) compared OT-SFC/FID with GC/FID for the analysis of fish oil FAEEs. Six different GC methods and three different SFC methods were tested and compared for the 39 identified components, including cholesterol in sand eel fish oil. Both GC and SFC methods showed good reproducibility within methods and fair agreement between methods. Columns of different polarity were used for the different methods. For the SFC separation of FAEEs in fish oil, the polar DB-225 (50% cyanopropylphenyl) proved optimal. A chromatogram of FAEEs from sand eel using this column is shown in Figure 11. In general, the SFC methods gave longer analysis times than the GC methods, but on the other hand, SFC enabled simultaneous determination of both FAEEs and cholesterol. Furthermore, SFC allowed lower operating temperatures than GC, which minimizes thermal degradation of polyunsaturated compounds.

Figure 11

Archaebacterial ether lipids were analyzed by OT-SFC/FID (72). The developed SFC method using SC-CO_2 as mobile phase and a SB phenyl-5 capillary column, enabled group separation of ether-containing lipids as glycolipid, polar lipid, and lipid-extracted residue fractions. A detection limit of 0.6 ng for a diether standard was obtained, which was far more sensitive than reported LC methods.

Capillary SFC was also used for analysis of oligomers of propoxylated glycerols and FA-esterified propoxylated glycerols (73). A nonpolar SB-methyl-100 column was used for separation and FID for detection. Separation depended mainly on the molecular weight or the number of the propylene oxide units.

4.5 Preparative SFC

The principles of preparative and analytical SFC are similar. However, larger columns are used in preparative SFC, which permit larger sample loading and faster flow rates. Compared to preparative LC, preparative SFC offers better resolution and faster separation but smaller maximum sample loading. For a given separation with a specified set of conditions, an increase in flow rate or sample loading will result in a poorer

separation but an improved production rate. Hence, there is an optimum sample loading or flow rate for an optimal separation that gives the highest production rate and solute purity (74).

The most important parameters to optimize in preparative SFC are column type, temperature, pressure, modifier type and concentration, flow rate and sample loading. The easiest (and most economical) way of performing the optimization study is to use an analytical-scale SFC system. For example, Alkio et al. (75) used 250 mm × 10 mm ODS columns to optimize the process parameters for the preparative purification of polyunsaturated FAE from tuna oil.

Dermaux et al. (76) fractionated TAGs in fish oil according to degree of unsaturation using a silver-ion packed column (Nucleosil 100-5 SA, 25 cm × 4.6 mm, loaded with silver ions). SC-CO_2 containing acetonitrile/isopropanol (6:1) as modifier was used as mobile phase and detection by UV at 210 nm. A chromatogram is shown in Figure 12. Eighteen fractions were collected, and each fraction was analyzed by capillary electro chromatography (CEC) with DAD as well as by MS. The chromatograms from CEC analysis were relatively simple, since in each fraction the TAGs differed only in CN. Since CEC separates TAGs based on their partition number (defined by PN=CN-2UN), the peak identities in the CEC chromatograms could be elucidated.

Figure 12

Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) ethyl esters were purified from tuna oil using preparative SFC on ODS columns (75). In order to obtain a cost-effective process, process variables were optimized so that the production rate per mass of stationary phase was at maximum, while product purity was maintained at a certain level. DHA and EPA ester concentrates of up to 95 and 50 wt% purity, respectively, were obtained using SC-CO_2 as mobile phase. The SFC operating cost was calculated to $550/kg of DHA and EPA ethyl ester concentrate.

5 Conclusions

In this chapter we have reported on the current status and application of SFC to lipid mixtures and solutes. SFC is a "natural" technique for such separations due to the

relatively high solubilities of lipid moieties in SC-CO_2 and similar fluids. This has resulted in numerous applications and separations as reported in this chapter. The major advantage of SFC compared to LC is the possibility of using one fluid in one single chromatographic run to separate lipids of widely differing molecular weights and polarities. This property makes the technique attractive in both analytical and preparative work. Another benefit with SFC is that both GC and LC type of detectors can be used, of which FID and MS detectors have clearly been the most popular ones in lipid analysis. Some of the applications discussed above also demonstrate the use of SFC for studying molecular weights and degree of unsaturation of lipid moieties. Obviously, SFC can be utilized for structural elucidations if a careful choice of column is made. Furthermore, SFC is a “green” technique, which does not use any organic solvents (or very small amounts). Relative to GC, SFC offers an option for less sample preparation or derivatization, extension of the fractionating range with respect to solute molecular weight, and superior resolution or application when applied to oligomeric mixtures and/or speciation of lipid classes. It is for the above reasons that SFC is a preferred and viable alternative to HPLC or GC.

A plethora of applications have been cited for both OT-SFC and PC-SFC, which demonstrate the versatility and flexibility of the technique. Analytical PC-SFC can serve a precursor or "scouting" technique for preparative SFC when applied to scaled up separations and engineering applications. Preparative SFC can save substantially on the use of organic solvents, offers more rapid separations relative to HPLC, and is an environmentally benign technique. SFC can also be utilized for physiochemical property measurements in support of SFC, or to measure solute-SF properties. However a discussion of these applications is beyond the scope of this present review.

6 Abbreviations

APCI Atmospheric pressure chemical ionization

API Atmospheric pressure ionization

CEC Capillary electro chromatography

CN Carbon number

DAD	Diode array detection
DAG	Diacylglycerol
ECD	Electron capture detection
ELSD	Evaporative light scattering detection
ESI	Electrospray ionization
FA	Fatty acid
FAE	Fatty acid ester
FAEE	Fatty acid ethyl ester
FAME	Fatty acid methyl ester
FFA	Free fatty acid
FID	Flame ionization detection
FT-IR	Fourier transform infrared detection
GC	Gas chromatography
HETP	Height equivalent of theoretical plates
HPLC	High performance liquid chromatography
IR	Infrared
LC	Liquid chromatography
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
NPD	Nitrogen phosphorus detection
OT	Open tubular
PC	Packed column
SC-CO_2	Supercritical carbon dioxide
SF	Supercritical fluid

SFC Supercritical fluid chromatography

SFE Supercritical fluid extraction

SPE Solid phase extraction

TAG Triacylglycerol

TLC Thin layer chromatography

TMS Trimethyl silylated

UN Unsaturation number

UV Ultra violet

VIS Visible

7 References

1. Clifford, T. (1998) *Fundamentals of Supercritical Fluids,* pp. 210, Oxford University Press, Oxford.
2. Bicchi, C., Binello, A., and Rubiolo, P. (2000) Packed Column SFC/UV Versus HPLC/UV Analysis of Valerenic Acids and Valepotriates in Extracts of Valeriana Officinalis L., *Phytochem Analysis 11,* 179-183.
3. Lee, M.L., and Markides, K.E. (1990) *Analytical Supercritical Fluid Chromatography and Extraction,* pp. 577, Chromatography Conferences Inc., Pravo, Utah.
4. King, J.W., Jr., H.H.H., and Lee, M.L. (1993) Analytical Supercritical Fluid Chromatography and Extraction, in *Physical Methods of Chemistry Series 2nd Ed.* (Rossiter, B.W., and Baetzold, R.C., eds), pp. 1-83, John Wiley & Sons, Inc, New York.
5. Lee, M.L., and Markides, K.E. (1986) Current Technological Challenges in Capillary Supercritical Fluid Chromatography, *J High Res Chromatog 9,* 652-656.
6. Giddings, J.C., Myers, M.N., McLaren, L., and Keller, R.A. (1968) High Pressure Gas Chromatography of Nonvolatile Species, *Science 162,* 67-73.
7. Wong, J.M., and Johnston, K.P. (1986) Solubilization of Biomolecules in Carbon Dioxide Based Supercritical Fluids, *Biotechnol. Progr. 2,* 30-39.
8. Friedrich, J.P., and Pryde, E.H. (1984) Supercritical CO2 Extraction of Lipid-Bearing Materials and Characterization of the Products, *J. Am. Oil Chem. Soc. 61,* 223-228.
9. Illes, V., Szalai, O., Then, M., Daood, H., and Perneczki, S. (1997) Extraction of Hiprose Fruit by Supercritical CO2 and Propane, *J. Supercrit. Fluids 10,* 209-218.

10. Karlsson, L., Jaremo, M., Emilsson, M., Mathiasson, L., and Jonsson, J.A. (1993) Retention in Coupled Capillary Column Supercritical-Fluid Chromatography with Lipids as Model Compounds, *Chromatographia 37,* 402-410.

11. Fields, S.M., Markides, K.E., and Lee, M.L. (1987) Retention in Capillary Supercritical Fluid Chromatography with Carbon-Dioxide Mobile Phases Containing Polar Modifiers, *J Chromatogr 406,* 223-235.

12. Lesellier, E., and Tchapla, A. (1999) Retention Behavior of Triglycerides in Octadecyl Packed Subcritical Fluid Chromatography with CO2/Modifier Mobile Phases, *Anal. Chem. 71,* 5372-5378.

13. Demirbuker, M., Hagglund, I., and Blomberg, L.G. (1992) Separation of Unsaturated Fatty-Acid Methyl-Esters by Packed Capillary Supercritical Fluid Chromatography - Comparison of Different Column Packings, *J Chromatogr 605,* 263-267.

14. Board, R., McManigill, D., Weaver, H., and Gere, D. (1983) The Use of Modifiers in Supercritical Fluid Chromatography with Carbon Dioxide, *Chemsa* 12, 21-12, 24.

15. Chester, T.L., Pinkston, J.D., and Raynie, D.E. (1994) Supercritical Fluid Chromatography and Extraction, *Anal. Chem. 66,* 106R-130R.

16. Chester, T.L., Pinkston, J.D., and Raynie, D.E. (1996) Supercritical Fluid Chromatography and Extraction, *Anal. Chem. 68,* 487R-514R.

17. Chester, T.L., Pinkston, J.D., and Raynie, D.E. (1998) Supercritical Fluid Chromatography and Extraction, *Anal. Chem. 70,* 301R-319R.

18. Lee, M.L., and Markides, K.E. (1987) Chromatography with Supercritical Fluids, *Science 235,* 1342-1347.

19. Kallio, H., Johansson, A., and Oksman, P. (1991) Composition and Development of Turnip Rapeseed (Brassica-Campestris) Oil Triacylglycerols at Different Stages of Maturation, *J Agr Food Chem 39,* 1752-1756.

20. Smith, R.H., Hyytiainen, M., Felipe, A.V., and Morris, P.M. (2001) High Resolution Packed Column Supercritical Fluid Chromatography of Fatty Acid Methyl Esters, *J Sep Sci 24,* 208-212.

21. France, J.E., Snyder, J.M., and King, J.W. (1991) Packed-Microbore Supercritical Fluid Chromatography with Flame Ionization Detection of Abused Vegetable-Oils, *J Chromatogr 540,* 271-278.

22. Staby, A., Borch-Jensen, C., Balchen, S., and Mollerup, J. (1994) Quantitative-Analysis of Marine Oils by Capillary Supercritical-Fluid Chromatography, *Chromatographia 39,* 697-705.

23. Rezaei, K., and Temelli, F. (2000) Lipase-Catalyzed Hydrolysis of Canola Oil in Supercritical Carbon Dioxide, *J Am Oil Chem Soc 77,* 903-909.

24. Berg, B.E., Lund, H.S., and Greibrokk, T. (1997) Separation and Quantification of Components of Edible Fat Utilizing Open Tubular Columns in SFC. Sample

Introduction by Direct Injection and SFE Coupled on-Line to SFC., *Chromatographia 44*, 399-404.

25. Artz, W.E., and Sauer, R.M. (1992) An Improved Micro-Extraction Cell for Supercritical Fluid Extraction and Chromatography of Fatty-Acids, *J Am Oil Chem Soc 69*, 309-313.

26. Bucherl, T., Eschler, M., Gruner, A., Palibroda, N., and Wolff, E. (1994) Direct Coupling of Capillary Supercritical-Fluid Chromatography with Double-Focusing High-Resolution Mass-Spectrometry, *HRC-J High Res Chrom 17*, 765-769.

27. Borch-Jensen, C., Staby, A., and Mollerup, J. (1993) Supercritical-Fluid Chromatographic Analysis of a Fish-Oil of the Sand Eel (Ammodytes Sp), *HRC-J High Res Chrom 16*, 621-623.

28. Manninen, P., Laakso, P., and Kallio, H. (1995) Separation of Gamma-Linolenic and Alpha-Linolenic Acid-Containing Triacylglycerols by Capillary Supercritical-Fluid Chromatography, *Lipids 30*, 665-671.

29. Manninen, P., Laakso, P., and Kallio, H. (1995) Method for Characterization of Triacylglycerols and Fat-Soluble Vitamins in Edible Oils and Fats by Supercritical-Fluid Chromatography, *J Am Oil Chem Soc 72*, 1001-1008.

30. Upnmoor, D., and Brunner, G. (1989) Investigation of Retention Behavior in Packed Column SFC, *Ber. Bunsen-Ges. Phys. Chem. 93*, 1009-1015.

31. Sakaki, K. (1993) Supercritical-Fluid Chromatographic-Separation of Fatty-Acid Methyl-Esters on Aminopropyl-Bonded Silica Stationary-Phase, *J Chromatogr 648*, 451-457.

32. Hannan, R.M., and Hill, H.H. (1991) Analysis of Lipids in Aging Seed Using Capillary Supercritical Fluid Chromatography, *J Chromatogr 547*, 393-401.

33. Ryerson, T.B., Barkley, R.M., and Sievers, R.E. (1994) Selective Chemiluminescence Detection of Sulfur-Containing-Compounds Coupled with Nitrogen-Phosphorus Detection for Gas-Chromatography, *J Chromatogr A 670*, 117-126.

34. Markides, K.E., Lee, E.D., Bolick, R., and Lee, M.L. (1986) Capillary Supercritical Fluid Chromatography with Dual-Flame Photometric Detection, *Anal Chem 58*, 740-743.

35. Strode, J.T.B., and Taylor, L.T. (1996) Optimization of Electron-Capture Detector When Using Packed-Column Supercritical Fluid Chromatography with Modified Carbon Dioxide, *J Chromatogr A 723*, 361-369.

36. Huang, M.X., Markides, K.E., and Lee, M.L. (1991) Evaluation of an Ion Mobility Detector for Supercritical Fluid Chromatography with Solvent-Modified Carbon-Dioxide Mobile Phases, *Chromatographia 31*, 163-167.

37. Meier, U., and Trepp, C. (1993) Calibration of Spectrophotometric Detectors for Supercritical Fluid Chromatography, *J Chromatogr 629*, 361-370.

38. Lundanes, E., and Greibrokk, T. (1985) Group Separation of Oil Residues by Supercritical Fluid Chromatography, *J Chromatogr 349,* 439-446.

39. Bernal, J.L., Jimenez, J.J., Rivera, J.M., Toribio, L., and delNozal, M.J. (1996) On-Line Solid-Phase Extraction Coupled to Supercritical Fluid Chromatography with Diode Array Detection for the Determination of Pesticides in Water, *J Chromatogr A 754,* 145-157.

40. Herbreteau, B., Salvador, A., Lafosse, M., and Dreux, M. (1999) SFC with Evaporative Light-Scattering Sfc Detection and Atmospheric-Pressure Chemical-Ionisation Mass Spectrometry for Methylated Glucoses and Cyclodextrins Analysis, *Analusis 27,* 706-712.

41. Senorans, F.J., Markides, K.E., and Nyholm, L. (1999) Determination of Tocopherols and Vitamin A in Vegetable Oils Using Packed Capillary Column Supercritical Fluid Chromatography with Electrochemical Detection, *J Microcolumn Sep 11,* 385-391.

42. Nomura, A., Yamada, J., Yarita, T., and Maeda, T. (1995) Supercritical-Fluid Chromatograms of Fuel Oils on ODS-Silica Gel Column Using Fluorescence, UV-Absorption, and Flame-Ionization Detectors, *J Supercrit Fluids 8,* 329-333.

43. Sjoberg, P.J.R., and Markides, K.E. (1999) Capillary Column Supercritical Fluid Chromatography-Atmospheric Pressure Ionisation Mass Spectrometry - Interface Performance of Atmospheric Pressure Chemical Ionisation and Electrospray Ionisation, *J Chromatogr A 855,* 317-327.

44. Calvey, E.M., McDonald, R.E., Page, S.W., Mossoba, M.M., and Taylor, L.T. (1991) Evaluation of SFC/FT-IR for Examination of Hydrogenated Soybean Oil, *J Agr Food Chem 39,* 542-548.

45. Huang, E.C., Jackson, B.J., Markides, K.E., and Lee, M.L. (1988) Direct Heated Interface Probe for Capillary Supercritical Fluid Chromatography Double Focusing Mass-Spectrometry, *Anal Chem 60,* 2715-2719.

46. Huang, E.C., Jackson, B.J., Markides, K.E., and Lee, M.L. (1988) Direct Coupling of Capillary Supercritical Fluid Chromatography to High-Resolution Mass-Spectrometry with Minimum Modification, *Chromatographia 25,* 51-54.

47. Sjoberg, P.J.R., and Markides, K.E. (1997) New Supercritical Fluid Chromatography Interface Probe for Electrospray and Atmospheric Pressure Chemical Ionization Mass Spectrometry, *J Chromatogr A 785,* 101-110.

48. Broadbent, J.K., Martincigh, B.S., Raynor, M.W., Salter, L.F., Moulder, R., Sjoberg, P., and Markides, K.E. (1996) Capillary Supercritical Fluid Chromatography Combined with Atmospheric Pressure Chemical Ionisation Mass Spectrometry for the Investigation of Photoproduct Formation in the Sunscreen Absorber 2-Ethylhexyl-P-Methoxycinnamate, *J Chromatogr A 732,* 101-110.

49. Tyrefors, L.N., Moulder, R.X., and Markides, K.E. (1993) Interface for Open-Tubular Column Supercritical-Fluid Chromatography Atmospheric-Pressure Chemical-Ionization Mass-Spectrometry, *Anal Chem 65,* 2835-2840.

50. King, J.W. (1990) Applications of Capillary Supercritical Fluid Chromatography - Supercritical Fluid Extraction to Natural Products, *J. Chromatogr. Sci. 28,* 9-14.

51. Staby, A., Borch-Jensen, C., Balchen, S., and Mollerup, J. (1994) Supercritical-Fluid Chromatographic Analysis of Fish Oils, *J Am Oil Chem Soc 71,* 355-359.

52. Borch-Jensen, C., Magnussen, M.P., and Mollerup, J. (1997) Capillary Supercritical Fluid Chromatographic Analysis of Shark Liver Oils, *J Am Oil Chem Soc 74,* 497-503.

53. Hayes, D.G., Kleiman, R., Weisleder, D., Adlof, R.O., Cuperus, F.P., and Derksen, J.T.P. (1995) Occurrence of Estolides in Processed Dimorphotheca Pluvialis Seed Oil, *Ind Crop Prod 4,* 295-301.

54. Temelli, F., King, J.W., and List, G.R. (1996) Conversion of Oils to Monoglycerides by Glycerolysis in Supercritical Carbon Dioxide Media, *J Am Oil Chem Soc 73,* 699-706.

55. Rezaei, K., and Temelli, F. (2001) On-Line Extraction-Reaction of Canola Oil Using Immobilized Lipase in Supercritical CO2, *J Supercrit Fluids 19,* 263-274.

56. Martinez, J.L., Rezaei, K., and Temelli, F. (2002) Effect of Water on Canola Oil Hydrolysis in an Online Extraction-Reaction System Using Supercritical CO2, *Ind Eng Chem Res 41,* 6475-6481.

57. Manninen, P., and Laakso, P. (1997) Capillary Supercritical Fluid Chromatography Atmospheric Pressure Chemical Ionization Mass Spectrometry of Triacylglycerols in Berry Oils, *J Am Oil Chem Soc 74,* 1089-1098.

58. Manninen, P., and Laakso, P. (1997) Capillary Supercritical Fluid Chromatography Atmospheric Pressure Chemical Ionization Mass Spectrometry of Gamma- and Alpha-Linolenic Acid Containing Triacylglycerols in Berry Oils, *Lipids 32,* 825-831.

59. Laakso, P., Manninen, P., Makinen, J., and Kallio, H. (1996) Postparturition Changes in the Triacylglycerols of Cow Colostrum, *Lipids 31,* 937-943.

60. Laakso, P., and Manninen, P. (1997) Identification of Milk Fat Triacylglycerols by Capillary Supercritical Fluid Chromatography Atmospheric Pressure Chemical Ionization Mass Spectrometry, *Lipids 32,* 1285-1295.

61. Demirbuker, M., Blomberg, L.G., Olsson, N.U., Bergqvist, M., Herslof, B.G., and Jacobs, F.A. (1992) Characterization of Triacylglycerols in the Seeds of Aquilegia-Vulgaris by Chromatographic and Mass-Spectrometric Methods, *Lipids 27,* 436-441.

62. Shen, Y.F., Huang, H.X., Liu, X.L., Wang, Q.H., and Zhou, L.M. (1994) Applications of Capillary Supercritical-Fluid Chromatography to the Characterization of Edible Oils and Traditional Chinese Medicines, *HRC-J High Res Chrom 17,* 74-76.

63. Klemann, L.P., Aji, K., Chrysam, M.M., Damelia, R.P., Henderson, J.M., Huang, A.S., Otterburn, M.S., Yarger, R.G., Boldt, G., and Roden, A. (1994) Random

Nature of Triacylglycerols Produced by the Catalyzed Interesterification of Short-Chain and Long-Chain Fatty-Acid Triglycerides, *J Agr Food Chem 42*, 442-446.

64. Lee, T.W., and Hastilow, C.I. (1999) Quantitative Determination of Triacylglycerol Profile of Structured Lipid by Capillary Supercritical Fluid Chromatography and High-Temperature Gas Chromatography, *J Am Oil Chem Soc 76*, 1405-1413.

65. Artz, W.E., Hansen, S.L., and Myers, M.R. (1997) Heated Fat-Based Oil Substitutes, Oleic and Linoleic Acid-Esterified Propoxylated Glycerol, *J Am Oil Chem Soc 74*, 367-374.

66. Hayes, D.G., and Kleiman, R. (1996) A Detailed Triglyceride Analysis of Lesquerella Fendleri Oil: Column Chromatographic Fractionation Followed by Supercritical Fluid Chromatography, *J Am Oil Chem Soc 73*, 267-269.

67. Hayes, D.G., and Kleiman, R. (1996) Supercritical Fluid Chromatographic Analysis of New Crop Seed Oils and Their Reactions, *J Am Oil Chem Soc 73*, 1691-1697.

68. Skovly, J., Lundanes, E., and Greibrokk, T. (1996) Determination of the Identity of by-Products in the Industrial Production of Saturated Fatty Acids, *J Am Oil Chem Soc 73*, 1297-1304.

69. Borch-Jensen, C., and Mollerup, J. (1996) Determination of Vernolic Acid Content in the Oil of Euphorbia Lagascae by Gas and Supercritical Fluid Chromatography, *J Am Oil Chem Soc 73*, 1161-1164.

70. Borch-Jensen, C., Jensen, B., Mathiasen, K., and Mollerup, J. (1997) Analysis of Seed Oil from Ricinus Communis and Dimorphoteca Pluvialis by Gas and Supercritical Fluid Chromatography, *J Am Oil Chem Soc 74*, 277-284.

71. Staby, A., Borch-Jensen, C., Mollerup, J., and Jensen, B. (1993) Flame Ionization Detector Responses to Ethyl-Esters of Sand Eel (Ammodytes Lancea) Fish-Oil Compared for Different Gas and Supercritical-Fluid Chromatographic Systems, *J Chromatogr 648*, 221-232.

72. Hedrick, D.B., Guckert, J.B., and White, D.C. (1991) Archaebacterial Ether Lipid Diversity Analyzed by Supercritical Fluid Chromatography - Integration with a Bacterial Lipid Protocol, *J Lipid Res 32*, 659-666.

73. Lu, X.J.J., Myers, M.R., and Artz, W.E. (1993) Supercritical Fluid Chromatographic Analysis of the Propoxylated Glycerol Esters of Oleic-Acid, *J Am Oil Chem Soc 70*, 355-360.

74. Bartle, K.D., Bevan, C.D., Clifford, A.A., Jafar, S.A., Malak, N., and Verrall, M.S. (1995) Preparative-Scale Supercritical-Fluid Chromatography, *J Chromatogr A 697*, 579-585.

75. Alkio, M., Gonzalez, C., Jantti, M., and Aaltonen, O. (2000) Purification of Polyunsaturated Fatty Acid Esters from Tuna Oil with Supercritical Fluid Chromatography, *J Am Oil Chem Soc 77*, 315-321.

76. Dermaux, A., Medvedovici, A., Ksir, M., Van Hove, E., Talbi, M., and Sandra, P. (1999) Elucidation of the Triglycerides in Fish Oil by Packed-Column Supercritical Fluid Chromatography Fractionation Followed by Capillary Electrochromatography and Electrospray Mass Spectrometry, *J Microcol Sep 11*, 451-459.

Figure Captions

Figure 1. Schematic of a phase diagram.

Figure 2. Chromatogram of the valerenic acid fraction of an extract of *V. officinalis* using **(A)** PC-SFC/UV and **(B)** HPLC/UV (2). The peaks were identified as follows: 6, valerenic acid; 7, hydroxyvalerenic acid; and 8, acetoxyvalerenic acid.

Figure 3. SC-CO_2 extraction of hiprose seed oil at different pressures and temperatures (9).

Figure 4. General schematic of SFC equipment for **(A)** OT-SFC and **(B)** PC-SFC.

Figure 5 Chromatogram of calophyllum oil using PC-SFC and subcritical CO_2/ACN/MeOH (93:6.3:0.7, v/v/v) as a mobile phase (12).

Figure 6 Chromatogram of components in a lipstick formulation (50).

Figure 7 SFC of FFAs, retinol, ergocalciferol, cholecalciferol, squalene, tocopherols, cholesterols, wax esters, diacylglycerols (DAGs), cholesteryl esters and mixed TAGs (22,51). Peak identification: **1.** retinol, **2.** squalene, **3.** δ-tocopherol, **4.** γ-tocopherol, **5.** ergocalciferol, **6.** α-tocopherol + cholecalciferol, **7.** palmityl palmitate, **8.** palmityl oleate + oleyl palmitate, **9.** stearyl oleate + oleyl oleate, **10.** arachidyl arachidate, **11.** 1-myristyl-2-oleyl-3-palmityl-*rac*-glycerol, **12.** cholesteryl palmitoleate, **13.** 1,2-dipalmityl-3-oleyl-*rac*-glycerol + 1,3-dipalmityl-2-oleylglycerol, **14.** cholesteryl vaccenate + cholesteryl oleate + cholesteryl linoleate, **15.** 1-palmityl-2,3-distearyl-*rac*-glycerol + 1-palmityl-2,3-dioleyl-*rac*-glycerol, **16.** cholesteryl eicosenoate, **17.** 1,2-distearyl-3-oleyl-*rac*-glycerol + 1,2-dioleyl-3-stearyl-*rac*-glycerol.

Figure 8 Group separation of FFAs, monoacylglycerols, DAGs and TAGs using OT-SFC/FID (54).

Figure 9 Separation of TAGs in buckthorn seed oil using a polar OT-SFC/FID system (29). Peak identification: **1.** 48:1, **2.** 50:1/48:2, **3.** 50:2/52:1, 48:3, **4.** 52:2/50:3, **5.** 52:3/54:2, 50:4, 56:1, **6.** 52:4/54:3, 56:2, **7.** 54:4/52:5, 56:3, **8.** 54:5/52:6, 56:4, **9.** 54:6/56:5, **10.** 54:7, **11.** 54:8, **12.** 54:9.

Figure 10 Chromatogram of FFAs and FA dimers on a Dionex SB-Biphenyl-30 column with FID (68).

Figure 11 SFC of sand eel FAEE mixture on a DB-225 column (71). For peak identification see reference (71).

Figure 12 Chromatogram of separation of sardine oil TAGs using a silver-ion packed column and UV detection at 210 nm (76).

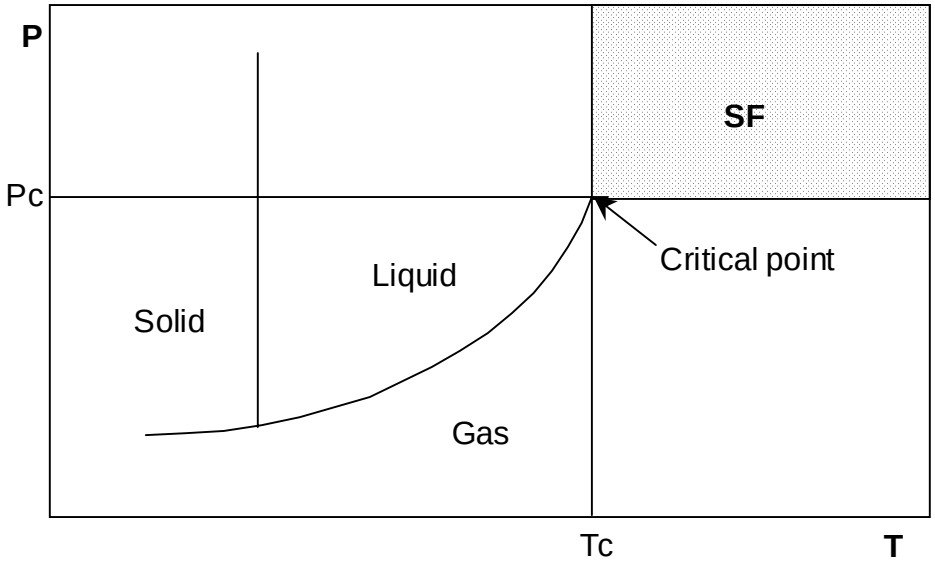
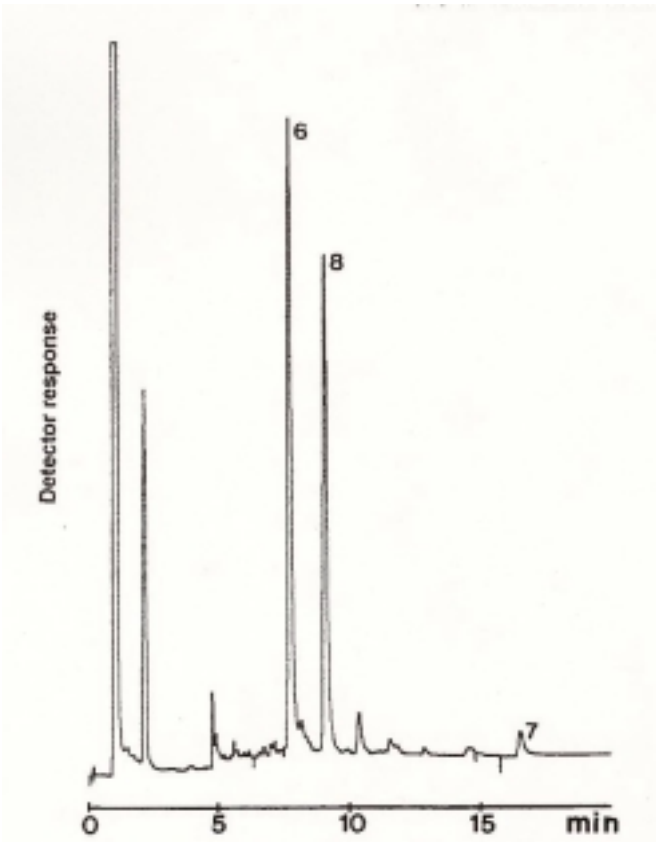
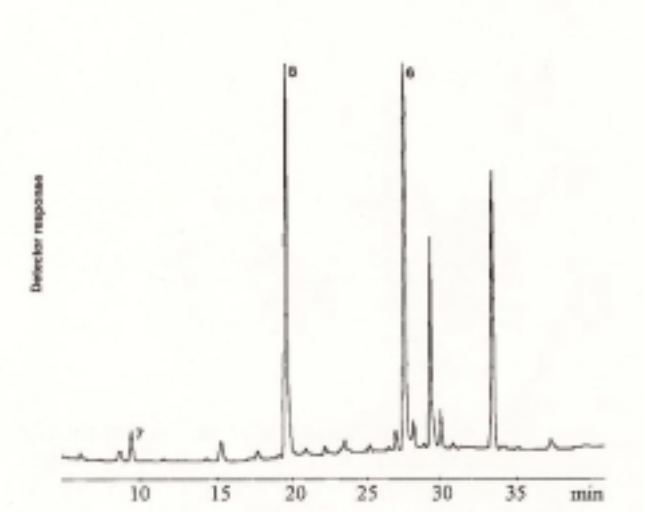


Figure 1



(A)



(B)

Figure 2

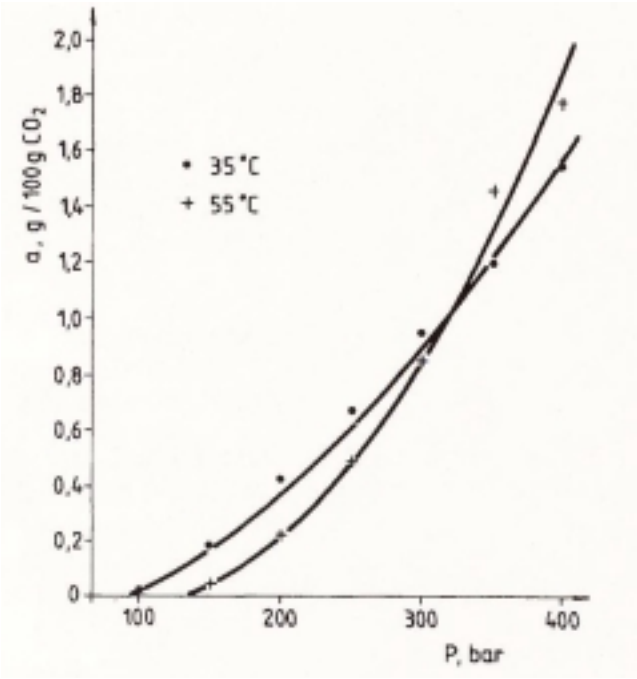
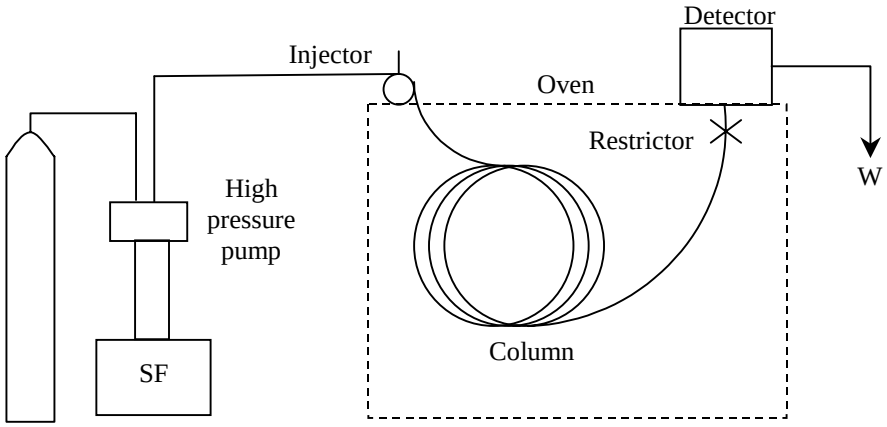
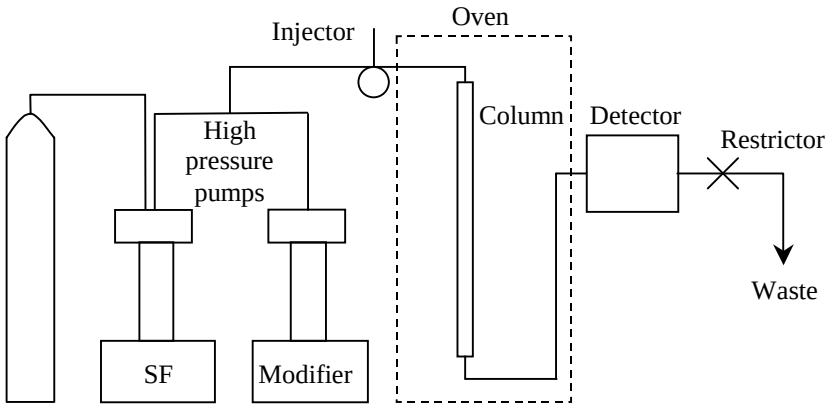

a, g / 100g CO_2
2,0
1,8
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0
• 35 °C
+ 55 °C
100
200
300
400
P, bar

Figure 3


Detector
Injector
Oven
Restrictor
W
High
pressure
pump
Column
SF

A


Oven
Injector
Column
Detector
Restrictor
High
pressure
pumps
Waste
SF
Modifier

B

Figure 4

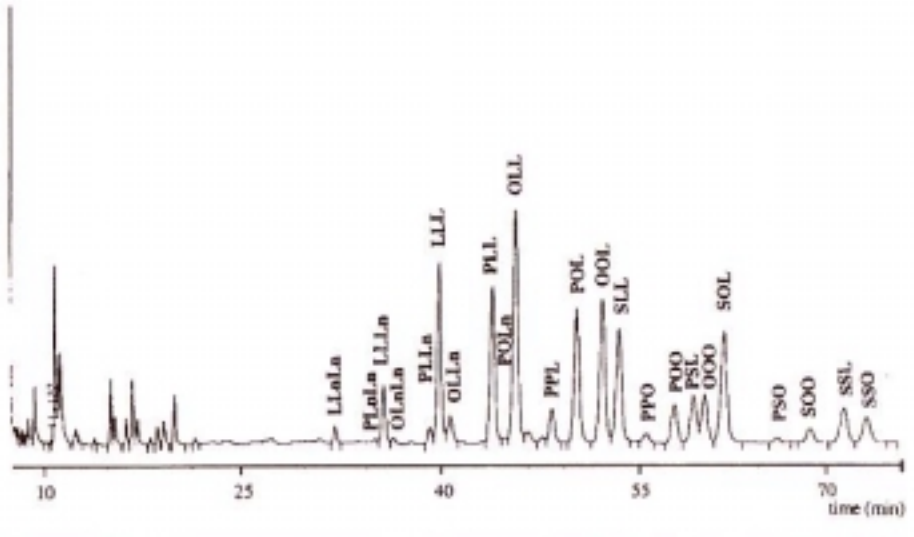

LLnLn
PLnLn
LLLn
OLnLn
PLLn
LLL
OLLn
PLL
POLn
OLL
PPL
POL
OOL
SLL
PPO
POO
PSL
OOO
SOL
PSO
SOO
SSL
SSO
10
25
40
55
70
time (min)

Figure 5

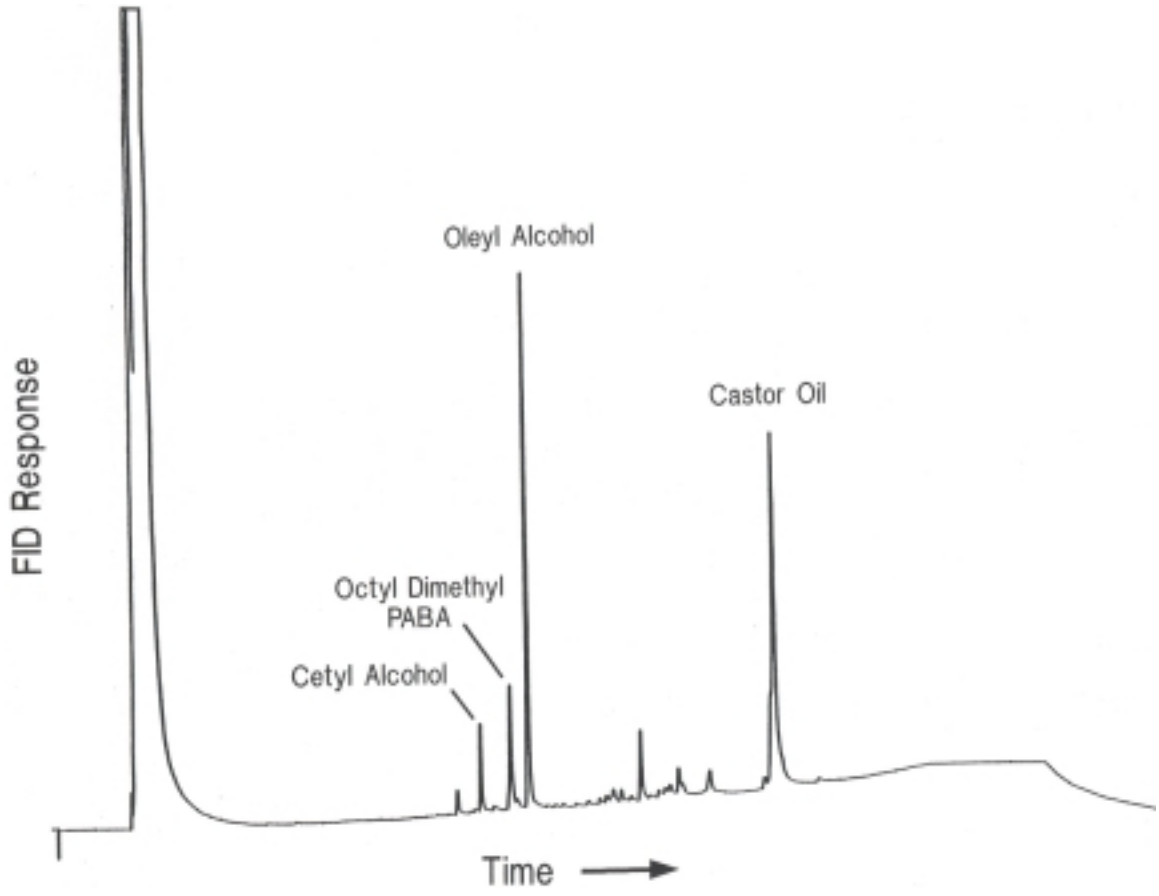

Oleyl Alcohol
Castor Oil
FID Response
Octyl Dimethyl
PABA
Cetyl Alcohol
Time

Figure 6

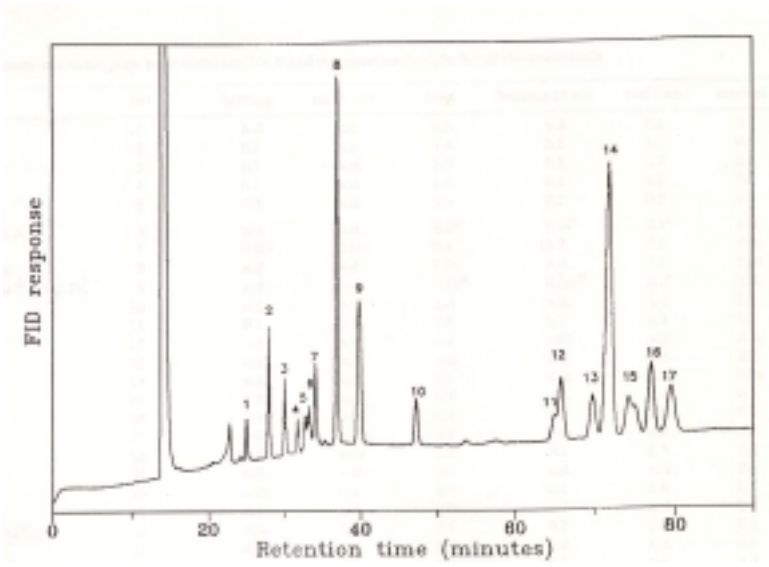

FID response
Retention time (minutes)
0
20
40
60
80
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17

Figure 7

Figure 8

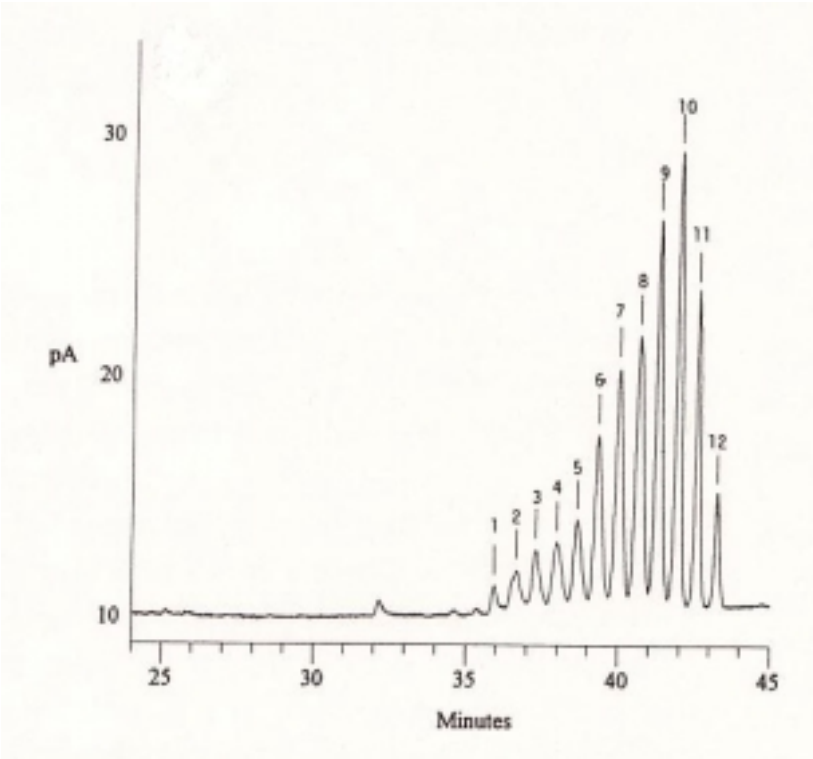


Figure 9

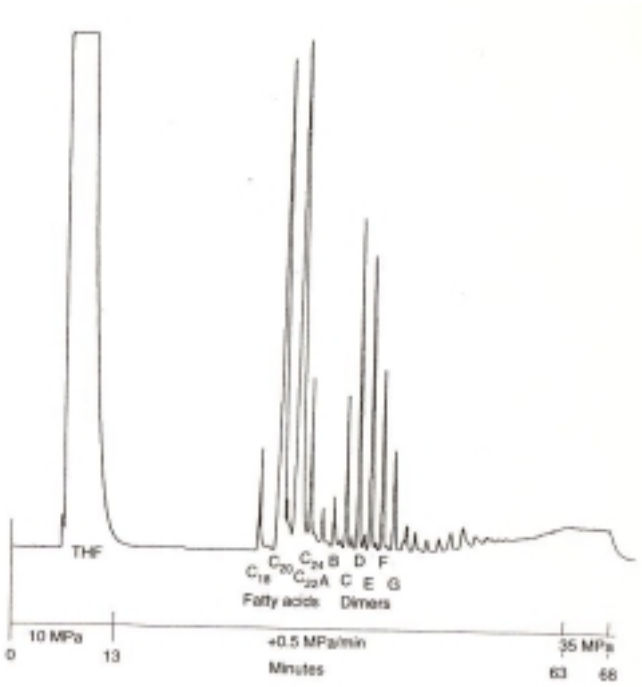

THF
C18 C20 C22 C24
A B C D E F G
Fatty acids
Dimers
10 MPa
+0.5 MPa/min
35 MPa
0
13
63
68
Minutes

Figure 10

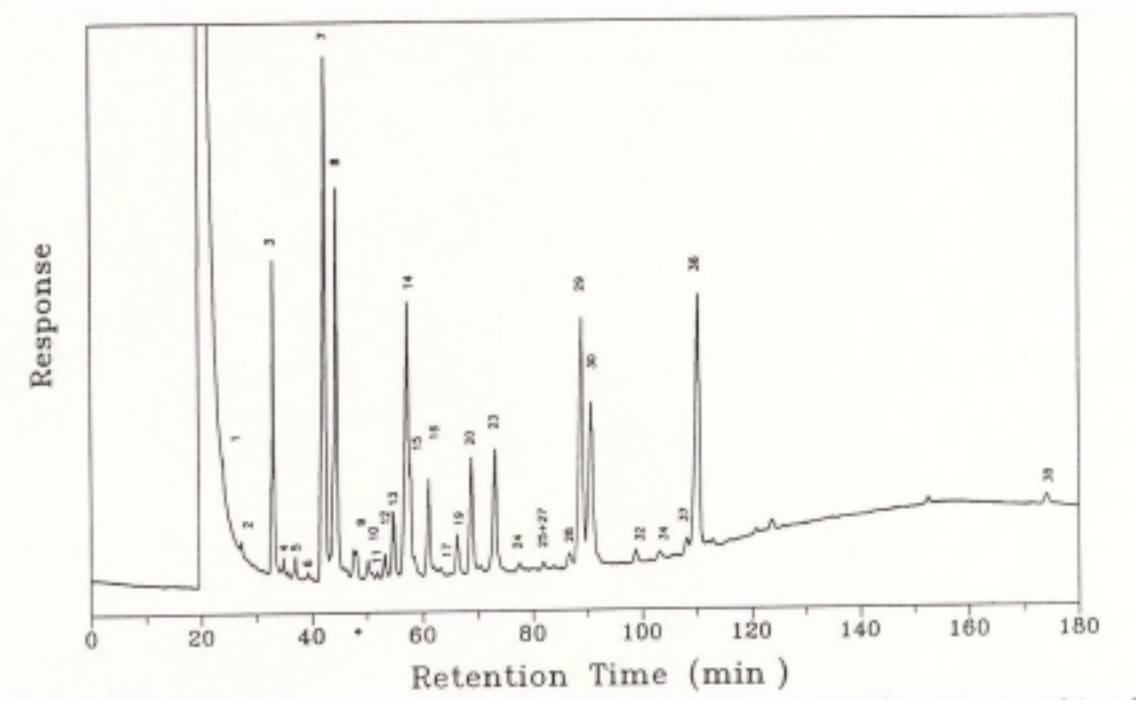


Figure 11

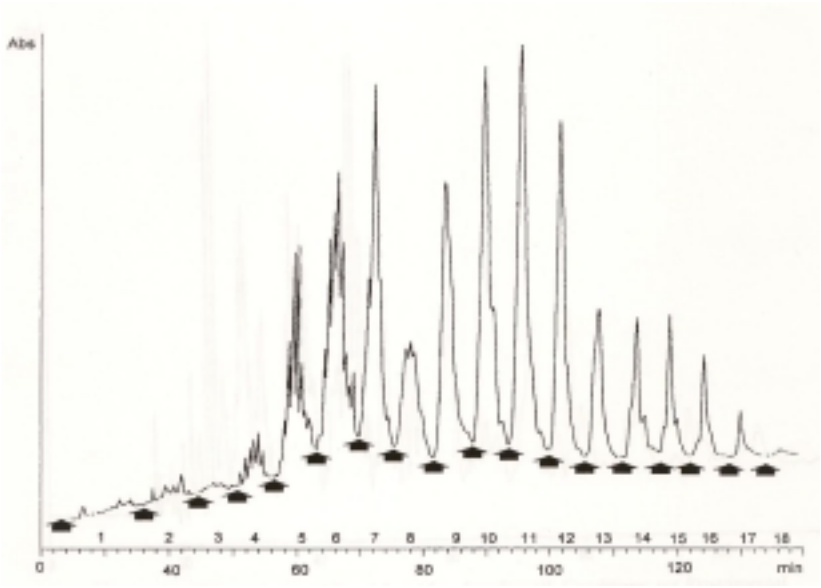


Figure 12

Table 1 Properties of some selected SFs (3).

Fluid	**T_c (°C)**	**P_c (atm)**	**ρ_c (g/mL)**
CO_2	31.3	72.9	0.47
H_2O	374	218	0.32
N_2O	36.5	72.5	0.45
NH_3	132.5	112.5	0.24
n-C_4H_{10}	196.6	33.3	0.23
n-C_5H_{12}	152.0	37.5	0.23
SF_6	45.5	37.1	0.74
Xe	16.6	58.4	1.10
CCl_2F_2	111.8	40.7	0.56
CHF_3	25.9	46.9	0.52

Table 2. SFC of lipid classes

Analyte and sample	Column dimensions	Stationary phase	Detection	Ref.
Cholesterol in fish oil	10 m × 50 μm i.d.	SB-Methyl-100	FID	(50)
Acylglycerols in heated vegetable oil	150 × 1 mm i.d.	Deltabond Octyl microbore	FID	(21)
Acylglycerols, FAs, vitamins, wax esters and steroids in marine oils	20 m × 100 μm i.d.	5% phenyl- 95% methyl polysiloxane, 0.1 μm	FID	(22,51, 52)
FFA, DAGs, TAGs and estolides in *D. pluvialis* seeds	10 m × 50 μm i.d.	SB-Methyl-100	FID	(53)
FFA and acylglycerols in soybean oil	10 m × 100 μm i.d.	Dionex SB-Octyl-50, 0.5 μm	FID	(54)
FFA and acylglycerols in canola oil	10 m × 50 μm i.d.	SB-Methyl-100, 0.25 μm	FID	(23,55)

Table 3. SFC of triacylglcyerols

Analyte and sample	Column dimensions	Stationary phase	Detection	Ref.
TAGs in rapeseed oil	100 mm × 1 mm i.d.	C18 5-μm	FID	(19)
TAGs in berry oils	10 m × 50 μm i.d.	25% cyanopropyl-75% methyl polysiloxane 0.25 μm	FID	(28)
TAGs in berry oils	10 m × 50 μm i.d.	25% cyanopropyl-25% phenyl-50% methyl polysiloxane, 0.25 μm	FID	(29)
TAGs in berry oils	10 m × 50 μm i.d.	25% cyanopropyl-25% phenyl-50% methyl polysiloxane, 0.25 μm	MS	(57,58)
TAGs in milk fat	10 m × 50 μm i.d.	SB-Octyl-50, 0.25 μm	FID	(59)
TAGs in milk fat	10 m × 50 μm i.d.	SB-Octyl-50, 0.25 μm	MS	(60)
TAGs in onion seed oil	20 m × 50 μm i.d.	SB-Methyl-100	FID	(32)
TAGs in *A. vulgaris* seed	330 mm × 250 μm i.d.	Nucleosil 5 SA, rinsed with silver nitrate	UV	(61)
TAGs in fish oil	20 m × 100 μm i.d.	5% phenyl- 95% methyl polysiloxane, 0.4 μm	FID	(27)
TAGs in fish oil	20 m × 100 μm i.d.	50% cyanopropyl-50% methyl polysiloxane, 0.1 μm	FID	(27)
TAGs in edible	16 m × 100	100% methyl polysiloxane	FID	(62)

and herbal oils	μm i.d.			
Structured lipids	100 mm × 1 mm	Deltabond Cyano 5-μm	FID	(63)
Structured lipids and TAGs in fish oil	10 m × 100 μm i.d.	SB-Methyl-100, 0.25 μm	FID	(64)
Heated triolein and trilinolein	17 m × 50 μm i.d.	25% cyanopropyl-25% phenyl-50% polymethyl siloxane 0.25 μm	FID	(65)
Heated EPG-08 oleate and linoleate	20 m × 50 μm i.d.	SB-Methyl-100, 0.25 μm	FID	(65)
TAGs in crop seeds	10 m × 50 μm i.d.	SB-Methyl-100	FID	(66,67)
TAGs in fish oil	250 mm × 4.6 mm i.d.	Nucleosil 100-5 SA with silver ions	UV 210nm	(76)

Table 4. SFC of FFAs and FA derivatives

Analyte and sample	Column dimensions	Stationary phase	Detection	Ref.
FFAs and FA dimers	10 m × 50 μm i.d.	Dionex SB-Biphenyl-30, 0.25 μm	FID and MS	(68)
Vernolic acid in *E. lagascae* oil	20 m × 100 μm i.d.	J&W Scientific DB-225, 0.2 μm	FID	(69)
Hydroxy FAs in *R. communis* and *D. pluvialis* oil	20 m × 100 μm i.d.	J&W Scientific DB-225, 0.2 μm	FID	(70)
FAMEs in fish oil	250 or 330 mm × 0.25 mm i.d.	Nucleosil 4 SA, Nucleosil 5 SB, and Superspher Si 60 4μm. Pure silica, silver nitrate loaded and permanganate treated	UV 210 nm	(13)
FAMEs in fish oil	150 × 4.6 mm i.d.	Cosmosil NH_2 10μm, Wakosil NH_2 5μm, and Super NH_2 10μm	UV 200 nm	(31)
FAEE in fish oil	20 m × 50 or 100 μm i.d.	25% cyanopropyl-25% phenyl-50% methyl polysiloxane, 0.1 μm, dimethylpolysiloxane, 0.2μm, and 5% phenyl-methyl polysiloxane, 0.4μm	FID	(71)
Ether lipids in archaic bacteria	10 m × 100 μm i.d.	5% phenyl-methyl polysiloxane, 0.5μm	FID	(72)
FA-esterified propoxylated glycerol	20 m × 50 μm i.d.	SB-Methyl-100, 0.25 μm	FID	(73)